

ADAMTS POLYPEPTIDES, NUCLEIC ACIDS ENCODING THEM, AND USES THEREOFField of Invention

The present invention relates to a member of the family of proteins known as ADAMTS.

5

Background Of The Invention

ADAMTS proteins exhibit characteristics of the ADAM (A Disintegrin And Metalloprotease) family of metalloproteases, and in addition contain a thrombospondin domain (TS). The prototypic ADAMTS was identified in mouse, found to be expressed in heart and kidney and upregulated by proinflammatory stimuli (K. Kuno et al., *Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as a inflammation associated gene*, 272 *Journal of Biological Chemistry* 556 (January 1997). To date nine members are recognized by the HUGO database (<http://www.gene.ucl.ac.uk/users/hester/adamts>). Members of this family have the ability to degrade aggrecan, a high molecular weight proteoglycan which provides cartilage with important mechanical properties and which is lost during the development of arthritis.

10

Aggrecanase activity has been demonstrated for several ADAMTS proteins (See, e.g., M.D. Tortorella, *Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins*, 284 *Science* 1664 (June 1999); I. Abbaszade, *Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family*, 274 *Journal of Biological Chemistry* 23443 (August 1999)). In addition to aggrecanase activity, ADAMTS-4 was shown to cleave another proteoglycan, brevican, found expressed predominately in the central nervous system (Matthews et al., *Brain-enriched hyaluronan binding (BEHAB)/Brevican cleavage in a glioma cell line is mediated by a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family member*, 275 *Journal of Biological Chemistry* 22695 (July 2000)). This activity was speculated to play a role in the invasiveness of glioma. Additional activities of ADAMTS-4 are proposed as its expression was induced in rat astrocytes treated with beta-amyloid, suggesting a role in Alzheimer's disease (Sato et al., *ADAMTS-4 is transcriptionally induced in beta-amyloid treated rat astrocytes*, 289 *Neuroscience Letters* 177 (2000)). Other ADAMTS proteins are reported to exhibit antiangiogenic (See, e.g. F. Vazquez et al., *METH-1, A human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with Angio-inhibitory activity*, 274 *Journal of Biological Chemistry* 23349 (August 1999)) and/or procollagen processing activities (A. Colige et al., *cDNA cloning and expression of bovine procollagen I N-proteinase: a new member of the superfamily of zinc-metalloproteinase with binding sites for cells and other matrix components*, 94 *Proceedings of the National Academy of Sciences of the United States of America* 2374 (March 1997)). Additional roles for ADAMTS-1 in fertility and organ development, particularly with respect to the urogenital system, were implicated by gene

15

20

25

30

35

knockout experiments in mice (Shindo et al., *ADAMTS-1: a metalloprotease-disintegrin essential for normal growth, fertility, and organ morphology and function*, 105 Journal of Clinical Investigation 1345 (May 2000))

ADAMTS proteins and ADAMTS protein agonists and antagonists have important  
5 therapeutic uses, including treatment of arthritis (osteoarthritis and rheumatoid arthritis),  
inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress  
syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's disease, organ  
transplant toxicity and rejection, cachexia, allergy, cancer (such as solid tumor cancer  
including colon, breast, lung, prostate, brain and hematopoietic malignancies including  
10 leukemia and lymphoma), tissue ulcerations, restenosis, periodontal disease, epidermolysis  
bullosa, osteoporosis, loosening of artificial joints implants, atherosclerosis (including  
atherosclerotic plaque rupture), aortic aneurysm (including abdominal aortic and brain aortic  
aneurysm), congestive heart failure, myocardial infarction, stroke, cerebral ischemia, head  
trauma, spinal cord injury, neurodegenerative diseases (acute and chronic), autoimmune  
15 disorders, Huntington's disease, Parkinson's disease, migraine, depression, peripheral  
neuropathy, pain, cerebral amyloid angiopathy, nootropic or cognition enhancement,  
amyotrophic lateral sclerosis, multiple sclerosis, ocular angiogenesis, corneal injury, macular  
degeneration, abnormal wound healing, burns, diabetic shock, infertility and other diseases  
characterized by metalloproteinase activity and/or characterized by mammalian adamalysin  
20 activity.

WO 00/53774 describes an ADAMTS protein having a sequence related to that of the  
present invention.

#### Summary Of The Invention

This invention relates to a novel ADAMTS protein, designated ADAMTS-SI, and to  
25 related polynucleotides and polypeptides. The invention also relates to production of the  
protein and polypeptides and to related assays. The invention further relates to methods for  
identifying substrates of the protein, for identifying inhibitors or activators of the protein, and to  
the use of the polypeptides or polynucleotides of the invention in diagnostic, biotherapeutic,  
and gene therapy methods.

30 In particular, the invention relates to an isolated polynucleotide molecule comprising a  
nucleotide sequence selected from the group consisting of:

- (a) a nucleotide sequence having at least 80% identity to a nucleotide sequence  
encoding ADAMTS-SI polypeptide of SEQ ID NO: 2 or a metalloproteinase or disintegrin  
domain, prodomain, or thrombospondin (TSP) domain thereof;
- 35 (b) a nucleotide sequence of at least 15 contiguous nucleotides that hybridizes  
under stringent conditions to the polynucleotide molecule of SEQ ID NO: 1; and
- (c) the complement of the nucleotide sequence of (a) or (b).

Such an isolated polynucleotide molecule, can, for example, comprise DNA or RNA.

In one embodiment, the isolated polynucleotide is at least 80% identical to SEQ ID NO: 1, and in another embodiment comprises the ADAMTS-SI polypeptide encoding sequence of SEQ ID NO: 1, or a sequence encoding a metalloproteinase or disintegrin domain, prodomain, or thrombospondin (TSP) domain thereof.

In a further embodiment, the invention relates to a polypeptide encoded by the isolated polynucleotide molecule of the invention. For example, the ADAMTS-SI polypeptide of the invention can comprise an amino acid sequence which is at least 80% identical to SEQ ID NO: 2 or a metalloproteinase or disintegrin domain, prodomain, or thrombospondin (TSP) domain thereof, or an amino acid sequence of at least about 10 contiguous amino acids of ADAMTS-SI. In a preferred embodiment, the polypeptide comprises SEQ ID NO: 2 or a metalloproteinase or disintegrin domain, prodomain, or thrombospondin (TSP) domain thereof.

In another aspect, the invention relates to an expression system comprising a DNA or RNA molecule, wherein the expression system is capable of producing an ADAMTS-SI polypeptide that comprises an amino acid sequence that has at least 80% identity with a polypeptide of SEQ ID NO: 2 or a metalloproteinase or disintegrin domain, prodomain, or thrombospondin (TSP) domain thereof, when said expression system is present in a compatible host cell. In one embodiment of this aspect of the invention, the expression system is capable of producing an ADAMTS-SI polypeptide encoded by a polynucleotide of the invention.

In another aspect, the invention relates to a host cell that comprises the expression system of the invention.

In another aspect, the invention relates to a process for producing an ADAMTS-SI polypeptide that comprises culturing a host cell of the invention under conditions sufficient for production of the polypeptide, and recovering the polypeptide from cell culture.

In another aspect, the invention relates to a process for producing a cell which produces an ADAMTS-SI polypeptide comprising transforming or transfecting a host cell with an expression system of the invention such that the host cell, under appropriate culture conditions produces the ADAMTS-SI polypeptide.

In another aspect, the invention relates to an antibody that is immunospecific for an ADAMTS-SI polypeptide of the invention. The invention also relates to antagonists, agonists, and substrates of the polypeptide of the invention.

In a further aspect, the invention relates to a method for treating a subject in need of altering activity or expression of ADAMTS-SI comprising administering to the subject a therapeutically effective amount of an agonist or antagonist of ADAMTS-SI.

In another aspect, the invention relates to a method for treating a subject in need of altering activity or expression of ADAMTS-SI comprising administering to the subject a polynucleotide of the invention in order to alter said activity or expression. The invention also relates to a method for treating a subject in need of altering activity or expression of  
5 ADAMTS-SI comprising administering to the subject a therapeutically effective amount of a polypeptide that competes with ADAMTS-SI for its ligand, substrate, or receptor.

The invention also relates to a process for diagnosing a disease or a susceptibility to a disease in a subject related to expression or activity of ADAMTS-SI in a subject comprising determining presence or absence of a mutation in a nucleotide sequence encoding ADAMTS-SI in the genome of the subject. Alternately, the invention relates to a process for diagnosing  
10 a disease or a susceptibility to a disease in a subject related to expression or activity of ADAMTS-SI in a subject comprising analyzing for presence or amount of ADAMTS-SI expression in a sample derived from the subject.

In another aspect, the invention relates to a method for identifying compounds which  
15 antagonize ADAMTS-SI comprising:

- (a) contacting a candidate compound with cells expressing an ADAMTS-SI polypeptide of the invention, or with cell membranes from cells expressing the ADAMTS-SI polypeptide, or the media conditioned by cells expressing the polypeptide, or a purified composition of said polypeptide; and
- 20 (b) determining inhibition of an ADAMTS-SI activity.

In an alternate embodiment, the invention relates to a method for identifying compounds which agonize ADAMTS-SI comprising:

- (a) contacting a candidate compound with cells expressing an ADAMTS-SI polypeptide of the invention, or with cell membranes from cells expressing the polypeptide, or  
25 media conditioned by cells expressing the polypeptide, or a purified composition of the polypeptide; and
- (b) determining stimulation of an ADAMTS SI activity.

In a further embodiment, the invention relates to a method for identifying compounds which bind to ADAMTS-SI comprising:

- 30 (a) contacting a candidate compound with cells expressing an ADAMTS-SI polypeptide of the invention, or with cell membranes from cells expressing the polypeptide, or the media conditioned by cells expressing the polypeptide, or a purified composition of the polypeptide; and
- (b) determining binding of the candidate compound to the polypeptide.

35 The invention also relates to a method for detecting a polynucleotide encoding ADAMTS-SI in a biological sample containing nucleic acid material comprising:

(a) hybridizing an isolated polynucleotide of the invention that is specific to ADAMTS-SI to the nucleic acid material of the biological sample, thereby forming a hybridization complex; and

(b) detecting the hybridization complex, wherein presence of the hybridization  
5 complex correlates with the presence of the polynucleotide encoding ADAMTS-SI in the biological sample.

In a further embodiment, the invention relates to a method for identifying a substrate for ADAMTS-SI comprising contacting a polypeptide comprising an enzymatically active polypeptide of the invention with a candidate substrate and determining either conversion of  
10 substrate to product or binding of the polypeptide to the substrate.

The invention also relates to a method for treating arthritis (osteoarthritis and rheumatoid arthritis), inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's disease, organ transplant toxicity and rejection, cachexia, allergy, cancer (such as solid tumor  
15 cancer including colon, breast, lung, prostate, brain and hematopoietic malignancies including leukemia and lymphoma), tissue ulcerations, restenosis, periodontal disease, epidermolysis bullosa, osteoporosis, loosening of artificial joints implants, atherosclerosis (including atherosclerotic plaque rupture), aortic aneurysm (including abdominal aortic and brain aortic aneurysm), congestive heart failure, myocardial infarction, stroke, cerebral ischemia, head  
20 trauma, spinal cord injury, neurodegenerative diseases (acute and chronic), autoimmune disorders, Huntington's disease, Parkinson's disease, migraine, depression, peripheral neuropathy, pain, cerebral amyloid angiopathy, nootropic or cognition enhancement, amyotrophic lateral sclerosis, multiple sclerosis, ocular angiogenesis, corneal injury, macular degeneration, abnormal wound healing, burns, or diabetic shock comprising administering a therapeutically effective amount of an agonist or antagonist, of ADAMTS-SI in combination  
25 with a pharmaceutically acceptable carrier.

The invention also relates to a method for treating arthritis (osteoarthritis and rheumatoid arthritis), inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's  
30 disease, organ transplant toxicity and rejection, cachexia, allergy, cancer (such as solid tumor cancer including colon, breast, lung, prostate, brain and hematopoietic malignancies including leukemia and lymphoma), tissue ulcerations, restenosis, periodontal disease, epidermolysis bullosa, osteoporosis, loosening of artificial joints implants, atherosclerosis (including atherosclerotic plaque rupture), aortic aneurysm (including abdominal aortic and brain aortic  
35 aneurysm), congestive heart failure, myocardial infarction, stroke, cerebral ischemia, head trauma, spinal cord injury, neurodegenerative diseases (acute and chronic), autoimmune disorders, Huntington's disease, Parkinson's disease, migraine, depression, peripheral

neuropathy, pain, cerebral amyloid angiopathy, nootropic or cognition enhancement, amyotrophic lateral sclerosis, multiple sclerosis, ocular angiogenesis, corneal injury, macular degeneration, abnormal wound healing, burns, or diabetic shock comprising administering a polypeptide of the invention in combination with a pharmaceutically acceptable carrier.

5           The invention also relates to a method for treating arthritis (osteoarthritis and rheumatoid arthritis), inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's disease, organ transplant toxicity and rejection, cachexia, allergy, cancer (such as solid tumor cancer including colon, breast, lung, prostate, brain and hematopoietic malignancies including  
10 leukemia and lymphoma), tissue ulcerations, restenosis, periodontal disease, epidermolysis bullosa, osteoporosis, loosening of artificial joints implants, atherosclerosis (including atherosclerotic plaque rupture), aortic aneurysm (including abdominal aortic and brain aortic aneurysm), congestive heart failure, myocardial infarction, stroke, cerebral ischemia, head trauma, spinal cord injury, neurodegenerative diseases (acute and chronic), autoimmune disorders, Huntington's disease, Parkinson's disease, migraine, depression, peripheral neuropathy, pain, cerebral amyloid angiopathy, nootropic or cognition enhancement,  
15 amyotrophic lateral sclerosis, multiple sclerosis, ocular angiogenesis, corneal injury, macular degeneration, abnormal wound healing, burns, infertility or diabetic shock comprising administering a polynucleotide of the invention in combination with a pharmaceutically acceptable carrier.  
20

          The invention further relates to a pharmaceutical composition for the treatment of arthritis (osteoarthritis and rheumatoid arthritis), inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's disease, organ transplant toxicity and rejection, cachexia,  
25 allergy, cancer (such as solid tumor cancer including colon, breast, lung, prostate, brain and hematopoietic malignancies including leukemia and lymphoma), tissue ulcerations, restenosis, periodontal disease, epidermolysis bullosa, osteoporosis, loosening of artificial joints implants, atherosclerosis (including atherosclerotic plaque rupture), aortic aneurysm (including abdominal aortic and brain aortic aneurysm), congestive heart failure, myocardial  
30 infarction, stroke, cerebral ischemia, head trauma, spinal cord injury, neurodegenerative diseases (acute and chronic), autoimmune disorders, Huntington's disease, Parkinson's disease, migraine, depression, peripheral neuropathy, pain, cerebral amyloid angiopathy, nootropic or cognition enhancement, amyotrophic lateral sclerosis, multiple sclerosis, ocular angiogenesis, corneal injury, macular degeneration, abnormal wound healing, burns, infertility  
35 or diabetic shock comprising a therapeutically effective amount of an agonist or antagonist, of ADAMTS-SI in combination with a pharmaceutically acceptable carrier.

The invention also relates to a pharmaceutical composition for the treatment of arthritis (osteoarthritis and rheumatoid arthritis), inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's disease, organ transplant toxicity and rejection, cachexia, allergy, cancer (such as solid tumor cancer including colon, breast, lung, prostate, brain and hematopoietic malignancies including leukemia and lymphoma), tissue ulcerations, restenosis, periodontal disease, epidermolysis bullosa, osteoporosis, loosening of artificial joints implants, atherosclerosis (including atherosclerotic plaque rupture), aortic aneurysm (including abdominal aortic and brain aortic aneurysm), congestive heart failure, myocardial infarction, stroke, cerebral ischemia, head trauma, spinal cord injury, neurodegenerative diseases (acute and chronic), autoimmune disorders, Huntington's disease, Parkinson's disease, migraine, depression, peripheral neuropathy, pain, cerebral amyloid angiopathy, nootropic or cognition enhancement, amyotrophic lateral sclerosis, multiple sclerosis, ocular angiogenesis, corneal injury, macular degeneration, abnormal wound healing, burns, infertility or diabetic shock comprising a polypeptide of the invention in combination with a pharmaceutically acceptable carrier.

The invention also relates to a pharmaceutical composition for the treatment of arthritis (osteoarthritis and rheumatoid arthritis), inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's disease, organ transplant toxicity and rejection, cachexia, allergy, cancer (such as solid tumor cancer including colon, breast, lung, prostate, brain and hematopoietic malignancies including leukemia and lymphoma), tissue ulcerations, restenosis, periodontal disease, epidermolysis bullosa, osteoporosis, loosening of artificial joints implants, atherosclerosis (including atherosclerotic plaque rupture), aortic aneurysm (including abdominal aortic and brain aortic aneurysm), congestive heart failure, myocardial infarction, stroke, cerebral ischemia, head trauma, spinal cord injury, neurodegenerative diseases (acute and chronic), autoimmune disorders, Huntington's disease, Parkinson's disease, migraine, depression, peripheral neuropathy, pain, cerebral amyloid angiopathy, nootropic or cognition enhancement, amyotrophic lateral sclerosis, multiple sclerosis, ocular angiogenesis, corneal injury, macular degeneration, abnormal wound healing, burns, infertility or diabetic shock comprising a polynucleotide of the invention in combination with a pharmaceutically acceptable carrier.

Brief Description Of The Drawings

FIG. 1 shows the complete polynucleotide sequence of ADAMTS-SI [SEQ ID NO:1].

FIG. 2 shows the substantially complete polypeptide sequence of ADAMTS-SI [SEQ ID NO:2].

FIG. 3 shows domains of the ADAMTS family of proteins, the sequences that correspond to those domains within the ADAMTS-SI polypeptide, and nucleic acid signature sequences.

FIG. 4 shows homology of the ADAMTS-SI polypeptide sequence of the metalloprotease domain aligned with those of other ADAMTS proteins.

FIG. 5 shows the results of a semi-quantitative PCR analysis of ADAMTS-SI expression and regulation by proinflammatory treatments in chondrocytes prepared from osteoarthritic cartilage.

FIG. 6 shows Western Blot analysis of purified recombinant ADAMTS-SI (amino acids 19-698) expressed in insect cells.

FIG. 7 shows Western Blot analysis of ADAMTS-SI expressed protein probed with Anti-V5-HRP.

FIG. 8 shows Western Blot analysis of ADAMTS-4 and ADAMTS-SI cleavage of aggrecanase substrate.

#### Detailed Description Of The Invention

We have found relatively high levels of polynucleotide encoding the ADAMTS-SI protein in a cDNA library prepared from osteoarthritic cartilage, and lower levels in cDNA libraries derived from human lung and brain. The expression of ADAMTS-SI in osteoarthritic cartilage, and its modulation by proinflammatory agents, are consistent with its role in the pathology of arthritic disease.

#### Definitions

The following definitions are provided to facilitate understanding of terms used herein.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of a Fab or other immunoglobulin expression library.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term "polynucleotide" also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example tritylated bases and unusual bases such as inosine. A variety of modifications have been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of



polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids  
5 joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. "Polypeptides" may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by  
10 chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given  
15 polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslational natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin,  
20 covalent attachment of a heme moiety, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and  
25 ubiquitination. See, for instance, *Proteins - structure and molecular properties*, 2nd Ed., T.E. Creighton, W.H. Freeman and Company, New York, 1993; F. Wold, *Posttranslational protein modifications: perspectives and prospects*, pgs. 1-12 in *Posttranslational covalent modification of proteins*, B.C. Johnson, Ed., Academic Press, New York, 1983; S. Seifter and S. England, *Analysis for protein modifications and nonprotein cofactors*, 182 *Methods of Enzymology* 626 (1990); S.I. Rattan et al., *Protein synthesis, posttranslational modifications, and aging*, 663 *Ann NY Acad Sci* 48 (1992).

"Variant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties.  
35 A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide

changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions and/or deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or a polypeptide may be naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

"Identity" is a measure of the identity of nucleotide sequences or amino acid sequences. In general, the sequences are aligned so that the highest order match is obtained. "Identity" per se has an art-recognized meaning and can be calculated using published techniques. See, for example, *Computational molecular biology*, A.M. Lesk, ed., Oxford University Press, New York, 1988; *Biocomputing: informatics and genome projects*, D.W. Smith, ed., Academic Press, New York, 1993; *Computer analysis of sequence data, part 1*, A.M. Griffin, and H.G. Griffin, eds., Humana Press, New Jersey, 1994; *Sequence analysis in molecular biology*, G. von Heinje, ed., Academic Press, 1987; and *Sequence analysis primer*, M. Gribskov and J. Devereux, eds., M Stockton Press, New York, 1991. While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans. Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCS program package; J. Devereux, et al., *A comprehensive set of sequence analysis programs for the VAX*, 12(1) *Nucleic Acids Research* 387 (January 1984); BLASTP; BLASTN; FASTA; S.F. Altschul et al., *Basic local alignment search tool*, 215(3) *Journal of Molecular Biology* 403 (October 1990). Among the methods stated above to determine identity, the preferred method is BLASTP.

As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 95% "identity" to a reference nucleotide sequence of FIG. 1, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence of FIG. 1. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total

nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

Similarly, by a polypeptide having an amino acid sequence having at least, for example, 95% "identity" to a reference amino acid sequence of FIG. 2, it is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of FIG. 2. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

#### **ADAMTS-SI Polypeptides**

In one aspect, the present invention relates to ADAMTS-SI polypeptides. The ADAMTS-SI polypeptides include the polypeptide of FIG. 2, as well as polypeptides comprising an amino acid sequence of FIG. 2, and polypeptides comprising an amino acid sequence that has at least 80% identity to that of FIG. 2, preferably at least 90% identity, more preferably at least 95% identity to FIG. 2, and most preferably at least 97-99% identity to FIG. 2.

The ADAMTS-SI polypeptides may be in the form of an unprocessed or partially processed precursor, or the "mature" protein, which may in turn be a part of a larger protein such as a fusion protein. The mature form should normally begin with or near amino acid 104 and continue to the carboxyl terminus. It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification or identification such as multiple histidine residues, or an additional sequence for stability during recombinant production.

Fragments of the ADAMTS-SI polypeptides are also included in the invention. A fragment is a polypeptide having an amino acid sequence that entirely is the same as part, but not all, of the amino acid sequence of the aforementioned ADAMTS-SI polypeptides. As with ADAMTS-SI polypeptides, fragments may be "free-standing," or comprised within a

larger polypeptide of which they form a part or region, most preferably as a single continuous region.

Preferred fragments include, for example, truncation polypeptides having the amino acid sequence of ADAMTS-SI polypeptides, except for deletion of a continuous series of residues that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus or deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Also preferred are fragments characterized by structural or functional attributes such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions. Also preferred are biologically active fragments. Biologically active fragments are those that mediate one or more ADAMTS-SI activities, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Most preferred are fragments that comprise one or more of the domains shown in FIG. 3. In particularly preferred embodiments, the fragment comprises the metalloproteinase domain, the disintegrin domain or the thrombospondin domain. In another embodiment, the polypeptide comprises amino acids 434 to 462 of SEQ ID NO: 2, which encompasses an extension of the zinc binding motif.

Such fragments are conventionally employed by themselves, or as part of fusion proteins. For example, expression vectors can be constructed that will express a fusion protein comprising a protein or polypeptide of the present invention. Such fusion proteins can be used, e.g., to raise antisera against the protein, to study the biochemical properties of the protein, to engineer a protein exhibiting different immunological or functional properties, to aid in the identification or purification, to improve the stability, of a recombinantly-expressed protein, or as therapeutic agents. Possible fusion protein expression vectors include but are not limited to vectors incorporating sequences that encode  $\beta$ -galactosidase and trpE fusions, maltose-binding protein fusions (pMal series; New England Biolabs), glutathione-S-transferase fusions (pGEX series; Pharmacia), polyhistidine fusions (pET series; Novagen Inc., Madison, WI), and thioredoxin fusions (pTrxFus; Invitrogen, Carlsbad, CA). As one example, the disintegrin domain or TSP domain, or a polypeptide comprising a variant or fragment thereof, may be administered alone, or as part of a fusion protein, to competitively inhibit in vivo or in vitro interactions with the native disintegrin domain or TSP domain. Methods are well-known in the art for constructing expression vectors encoding these and other fusion proteins.

Variants of the defined sequence and fragments also form part of the present invention. Preferred variants are those that vary from the referents by conservative amino

acid substitutions, i.e., those that substitute a residue with another of like characteristics. Typical conservative substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; among the basic residues Lys and Arg; and among the aromatic residues Phe and Tyr. Particularly preferred are variants in which several, 5 to 10, 1 to 5, or 1 to 2 amino acids are substituted, deleted, or added in any combination.

The ADAMTS-SI polypeptides of the invention can be prepared in any suitable manner. The polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, and polypeptides produced by a combination of these methods. These methods are well understood in the art.

Another embodiment of the present invention is an isolated ADAMTS-SI polypeptide. An isolated polypeptide is one that has been substantially removed from its natural milieu. As isolated ADAMTS-SI polypeptide can, for example, be obtained from its natural source, be produced using recombinant technology, or be synthesized chemically. An isolated ADAMTS-SI polypeptide can be full-length ADAMTS-SI polypeptide, the predicted mature form processed by furin cleavage of the prodomain (amino acid 292 with the predicted mature form beginning FLSY...), or any homologue of such a polypeptide, such as an ADAMTS-SI polypeptide in which amino acids have been deleted, inserted, inverted, substituted and/or derivatized (e.g., by glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitoylation, amidation and/or addition of glycosylphosphatidyl inositol). A homologue of an ADAMTS-SI polypeptide is a polypeptide having an amino acid sequence that is sufficiently similar to a natural ADAMTS-SI polypeptide amino acid sequence that a nucleic acid sequence encoding the homologue is capable of hybridizing under stringent conditions to a nucleic acid sequence encoding the natural ADAMTS-SI polypeptide amino acid sequence disclosed herein. As used herein, "stringent hybridization conditions" refers to hybridization to filter-bound DNA in 5XSSC, 5X Denhardt's, 1% SDS, and 100ug/ml denatured salmon sperm DNA at 65°C, and washing in 0.1xSSC/0.1% SDS at 65°C (see Ausubel *et al.* (eds.), 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3). A homologue of ADAMTS-SI polypeptide also includes a polypeptide having an amino acid sequence that is sufficiently cross-reactive such that the homologue has the ability to elicit an immune response against at least one epitope of naturally-occurring ADAMTS-SI polypeptide. Preferably the homologue retains one or more biological activities of ADAMTS-SI.

The minimal length of a protein homologue of the present invention is sufficient to be encoded by a nucleic acid molecule capable of forming a stable hybrid with the complementary sequence of a nucleic acid molecule encoding the corresponding natural protein. As such, the size of the nucleic acid molecule encoding such a protein homologue is

dependent on nucleic acid composition, percent homology between the nucleic acid molecule and complementary sequence, as well as upon hybridization conditions per se (e.g., temperature, salt concentration and formamide concentration). The minimal size of such nucleic acid is typically at least about 12 to about 15 nucleotides in length if the nucleic acid molecules are GC-rich and at least about 15 to about 17 bases in length if they are AT-rich. The minimal size of a nucleic acid molecule used to encode an ADAMTS-SI protein homologue of the present invention is from about 20 to about 25 nucleotides in length. There is no limit, other than a practical limit, on the maximal size of such a nucleic acid molecule in that the nucleic acid molecule can include a portion of a gene, an entire gene, or multiple genes, or portions thereof. In one embodiment, the minimal size of an ADAMTS-SI protein homologue of the present invention is from 10, more preferably 12, even more preferably 25, amino acids in length. In another embodiment, a polypeptide of the invention comprises an amino acid sequence of more than about 10, or 25, preferably more than 75, more preferably more than 100, amino acids that is identical to an amino acid sequence of SEQ ID NO: 2. Preferred protein or polypeptide sizes depend on whether a full-length, multivalent protein (i.e., fusion protein having more than one domain each of which has a function), or a functional portion of such a protein is desired. Functional portions are obtainable based on the domains described herein, knowledge in the art concerning such domains and known assays for such domain, or its functional activity. Useful protein fragments or other polypeptides can also be screened for based on antigenic cross-reactivity with the ADAMTS-SI protein of SEQ ID NO: 2.

ADAMTS-SI protein homologues of the invention include allelic variations of the natural gene encoding the ADAMTS-SI protein. A "natural" gene is that found most often in nature. ADAMTS-SI protein homologues can be produced using techniques known in the art, including, but not limited to, direct modifications to a gene encoding a protein using, for example, classic or recombinant DNA techniques to effect random or targeted mutagenesis.

In another embodiment, an ADAMTS-SI polypeptide of the present invention comprises a portion of the ADAMTS-SI polypeptide disclosed herein, this portion having a molecular weight of about 25 kD (determined by Tris-glycine SDS-PAGE and resolved using methods standard in the art).

The present invention, encompasses the ADAMTS-SI proteins that have undergone posttranslational modification. Such modification can include, for example, glycosylation (e.g., including addition of N-linked and/or O-linked oligosaccharides) or posttranslational conformational changes or posttranslational deletions.

Based on the 29-36% identity in the metalloprotease domain of ADAMTS-SI as compared to other ADAMTS family members, ADAMTS-SI may have one or more proteolytic activities (e.g. collagenase, aggrecanase, procollagen protease) as well as anti-angiogenic

activities that may or may not require the presence of the thrombospondin domains. See, FIGS. 3 and 4. These possible activities of ADAMTS-SI can be tested using techniques known to those skilled in the art. See, e.g., P.D. Brown et al., *Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines*, 50(19) *Cancer Research* 6184 (October 1990); F. Vazquez et al., *METH-1 a human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with angio-inhibitory activity*, 274 *The Journal of Biological Chemistry* 23349 (Aug. 1999); E.C. Arner et al., *Generation and characterization of aggrecanase*, 274 *The Journal of Biological Chemistry* 6594 (Mar. 1999); A. Colige et al., *cDNA cloning and expression of bovine procollagen I N-proteinase: A new member of the superfamily of zinc-metalloproteinases with binding sites for cell and other matrix components* 94 *Proceedings of the National Academy of Sciences (USA)* 2374 (March 1997).

#### ADAMTS-SI Polynucleotides

Another aspect of the invention relates to ADAMTS-SI polynucleotides. ADAMTS-SI polynucleotides include isolated polynucleotides which encode the ADAMTS-SI polypeptides and fragments, and polynucleotides closely related thereto. More specifically, ADAMTS-SI polynucleotides of the invention include a polynucleotide comprising the nucleotide sequence set forth in FIG. 1 encoding a ADAMTS-SI polypeptide of FIG. 2, and a polynucleotide having the particular sequence of FIG. 1. ADAMTS-SI polynucleotides further include a polynucleotide comprising a nucleotide sequence that has at least 80% identity to a nucleotide sequence encoding the ADAMTS-SI polypeptide of FIG. 2, and a polynucleotide that is at least 80% identical to the polynucleotide sequence of FIG. 1. In this regard, polynucleotides at least 90% identical are particularly preferred, and those with at least 95% are especially preferred. Furthermore, those with at least 97% are highly preferred and those with at least 98-99% are most highly preferred, with at least 99% being the most preferred.

In one embodiment, the nucleic acid molecule of the invention has a nucleotide sequence has between 1 and 50, more preferably between 1 and 25, and most preferably between 1 and 5 nucleotides inserted, deleted, or substituted with respect to the sequence of SEQ ID NO: 1.

ADAMTS-SI polynucleotides of the invention also encompass nucleotide sequences which have sufficient identity to the nucleotide sequence contained in FIG. 1 to hybridize under conditions useable for amplification or for use as a probe or marker for ADAMTS-SI. Such sequences are typically 15 to 25 nucleotides in length with a target of 50% GC content and useful in PCR amplification or oligonucleotide hybridization methods well known to those skilled in the art. (See, e.g., *Promega Protocols and Applications Guide, Third Edition, (1996), ISBN 1-8822474-57-1*).

In one embodiment, the isolated nucleic acid molecule comprises a fragment of SEQ ID NO: 1 that is specific for ADAMTS-SI, i.e., specifically acts as a probe for SEQ ID NO: 1. The fragment may be at least, e.g., 15, 25, 35, 45 or 75 nucleotides in length.

5 Another embodiment of the present invention is an isolated nucleic acid molecule capable of hybridizing, under stringent conditions, with ADAMTS-SI polypeptide gene (FIG. 1) encoding an ADAMTS-SI protein of the present invention.

An isolated nucleic acid of the invention can include DNA, RNA or derivatives of either DNA or RNA.

10 An isolated nucleic acid molecule of the present invention can be obtained from its natural source either as an entire (i.e., complete) gene or a portion thereof capable of forming a stable hybrid with that gene. As used herein, the phrase "at least a portion of" an entity refers to an amount of the entity that is at least sufficient to have functional aspects of that entity. For example, at least a portion of a nucleic acid sequence, as used herein, is an amount of a nucleic acid sequence capable of forming a stable hybrid with a particular desired  
15 gene (e.g., ADAMTS genes) under stringent hybridization conditions. An isolated nucleic acid molecule of the present invention can also be produced using recombinant technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Isolated ADAMTS-SI protein nucleic acid molecules include natural nucleic acid molecules and homologues thereof, including, but not limited to natural allelic variants and modified nucleic  
20 acid molecules in which nucleotides have been inserted, deleted, substituted, and/or inverted in a manner that does not substantially interfere with the nucleic acid molecule's ability to encode an ADAMTS-SI protein of the present invention or to form stable hybrids under stringent conditions with natural nucleic acid molecule isolates encoding an ADAMTS-SI protein.

25 The invention also provides polynucleotides that are complementary to ADAMTS-SI polynucleotides described above.

#### **Expression of ADAMTS-SI**

In one embodiment, an isolated ADAMTS-SI protein of the present invention is produced by culturing a recombinant cell capable of expressing the protein under conditions  
30 effective to produce the protein, and recovering the protein. Preferred cells include bacterial (e.g., *E. coli*), yeast (e.g., *Pichia*), insect (e.g., SF9) or mammalian cells (e.g., CHO, Cos 7, and HEK 293). The recombinant cell is capable of expressing the ADAMTS-SI protein and is produced by transforming a host cell with one or more nucleic acid molecules of the present invention. Such recombinant cells are part of the present invention. Suitable transformation  
35 techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption and protoplast fusion. Recombinant cells of the invention may remain unicellular or may grow into a tissue organ or a multicellular organism. Nucleic acid



molecules of the present invention used to transformed cells according to conventional techniques can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

5           Suitable host cells for transforming a cell include any cell capable of producing ADAMTS-SI proteins of the present invention after being transformed with at least one nucleic acid molecule of the present invention. Host cells can be either untransformed cells or cells that are already transformed with at least one nucleic acid molecule the present invention. Suitable host cells include bacterial, fungal (including yeast), insect, animal and plant cells.

10           The present invention also encompasses a recombinant vector which comprises a polynucleotide of the present invention inserted into a vector capable of delivering the polynucleotide into a host cell. Such a vector normally contains heterologous nucleic acid sequences, for example nucleic acid sequences that are not naturally found adjacent to ADAMTS-SI protein nucleic acid molecules of the present invention. The vector can be either  
15           DNA or RNA, and either prokaryotic or eukaryotic, and is typically a virus or a plasmid. Recombinant vectors can be used in cloning, sequencing, and/or otherwise manipulating or expressing ADAMTS-SI polynucleotides of the present invention.

          In one embodiment of the invention, a recombinant cell is produced by transforming a host cell with one or more recombinant molecules, each comprising one or more  
20           polynucleotide molecules of the present invention operatively linked to an expression vector containing one or more transcription control sequences. The phrase "operatively linked" refers to a nucleic acid molecule inserted into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, the phrase "expression vector" refers to a DNA or RNA vector that is capable of transforming a  
25           host cell and of effecting expression of a specified nucleic acid molecule.

          Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can be either prokaryotic or eukaryotic, and are typically viruses or plasmids. Expression vectors of the present invention include vectors that effect direct gene  
30           expression in bacterial, fungal, insect, animal, and/or plant cells. Nucleic acid molecules of the present invention can be operatively linked to expression vectors containing regulatory sequences such as promoters, operators, repressors, enhancers, termination sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of nucleic acid molecules. Transcription control sequences that can be used in the present invention include those capable of  
35           controlling the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcriptional initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control

sequences include those that function in one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in bacterial yeast and mammalian cells, such as, but not limited to, tac, lac, trp, trc, oxy-pro, omp/lpp, rmB, 5 bacteriophage lambda ( $\lambda$ ) (such as  $\lambda_P$  and  $\lambda P_R$  and fusions that include such promoters), bacteriophage T7, T7lac, bacteriophage T3, bacteriophage SP6, bacteriophage SPO1, metallothionein, alpha mating factor, baculovirus, vaccinia virus, herpesvirus, poxvirus, adenovirus, simian virus 40, retrovirus action, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences, as well as other 10 sequences capable of controlling gene expression in prokaryotic or eukaryotic cells. Additional suitable transcription control sequences include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (e.g., promoters inducible by interferons or interleukins). Transcription control sequences useful in practicing the present invention include naturally occurring sequences associated with DNA encoding an ADAMTS- 15 SI protein.

Preferred nucleic acid molecules for insertion into an expression vector include nucleic acid molecules encoding at least a portion of an ADAMTS-SI protein, or a homologue thereof. Expression vectors of the present invention may also contain fusion sequences, e.g., as discussed above, which allow expression of nucleic acid molecules of the present 20 invention as fusion proteins. Inclusion of a fusion sequence in an ADAMTS-SI nucleic acid molecule of the present invention can enhance stability during production, storage, or use of the protein encoded by the nucleic acid molecule. Furthermore, a fusion segment can simplify purification of an ADAMTS-SI protein, enabling purification by affinity chromatography (for example see FIG. 6). Fusion segments can be of any size that affords the desired 25 function (e.g., increased stability and/or easier purification). It is within the scope of the present invention to use one or more fusion segments. Fusion segments can be joined to amino and/or carboxyl termini of the ADAMTS-SI protein or polypeptide of the present invention. Linkages between fusion segments and ADAMTS-SI proteins can be constructed to be susceptible to cleavage to enable straightforward recovery of the ADAMTS-SI proteins. 30 Fusion proteins are preferably produced by culturing a recombinant cell transformed with nucleic acid sequences that encode the fusion segment attached to either the carboxyl and/or amino terminal end of a ADAMTS-SI polypeptide of the invention.

The present invention includes recombinant cells resulting from transformation with a nucleic acid molecule of the present invention. Preferred recombinant cells are transformed 35 with a nucleic acid molecule that encodes at least a portion of an ADAMTS-SI protein, or a homologue thereof. Amplifying the copy number of nucleic acid sequences of the invention can be accomplished by increasing the copy number of the nucleic acid sequence in the cell's

genome or by introducing additional copies of the nucleic acid sequence by transformation. Copy number amplification is conducted in a manner such that greater amounts of enzyme are produced, leading to enhanced conversion of substrate to product. Transformation can be accomplished using any process by which nucleic acids are transformed into cells to  
5 enhance enzyme synthesis. Prior to transformation, the nucleic acid sequence can, if desired, be manipulated to encode an enzyme having a higher specific activity.

In accordance with the present invention, recombinant cells are used to produce an ADAMTS-SI protein of the present invention by culturing such cells under conditions effective to produce such a protein, and the protein recovered. Effective conditions include, but are not  
10 limited to, appropriate media, bioreactor, temperature, pH and oxygen conditions that permit protein production. Suitable media are typically aqueous and comprise assimilable carbohydrate, nitrogen and phosphate sources, as well as appropriate salts, minerals, metals and other nutrients, such as vitamins. The medium may comprise complex nutrients, or may be minimal.

Cells of the present invention can be cultured in conventional fermentation bioreactors, which include, but are not limited to, batch, fed-batch, cell recycle, and continuous fermentors. Culturing can also be conducted in shake flasks, test tubes, microtiter  
15 dishes, and petri plates. Culturing is carried out at a temperature, pH and oxygen content appropriate for the recombinant cell. Such culturing is within the expertise of one of ordinary skill in this art.

Depending on the vector and host system used for production, resultant ADAMTS-SI proteins may either remain within the recombinant cell or be secreted into the fermentation medium. "Recovering the protein" according to the invention may involve simply collecting  
20 the fermentation medium or cells containing the protein and need not include additional steps of separation or purification. ADAMTS-SI proteins of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography (FIG. 6), ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, chromatofocusing and differential solubilization.

In addition, an ADAMTS-SI protein of the present invention can be produced by isolating the ADAMTS-SI protein from cells expressing the ADAMTS-SI protein recovered from transgenic animal, or from fluid, such as milk, recovered from such an animal. An isolated protein or polypeptide of the present invention can be used to formulate a therapeutic  
30 composition as discussed further below.

#### **Antibodies to ADAMTS-SI**

The present invention also includes antibodies capable of selectively binding to an ADAMTS-SI protein or polypeptide of the present invention. Polyclonal populations of anti-

ADAMTS-SI antibodies can be contained in an ADAMTS-SI antiserum. Binding can be measured using a variety of methods known to those skilled in the art including immunoblot assays, immunoprecipitation assays, enzyme immunoassays (e.g., ELISA), radioimmunoassay, immunofluorescent antibody assays and immunoelectron microscopy; see, for example, Sambrook et al., *Molecular cloning: a laboratory manual*, Cold Spring Harbor Labs Press, 1989.

Antibodies of the present invention can be either monoclonal or polyclonal antibodies and can be prepared using techniques standard in the art. Antibodies of the present invention include functional equivalents such as antibody fragments and genetically-engineered antibodies, including single chain antibodies that are capable of selectively binding to at least one of the epitopes of the protein used to obtain antibodies. Preferably, antibodies are raised in response to proteins that are encoded, at least in part, by an ADAMTS-SI nucleic acid molecule.

#### **Identification of ADAMTS-SI substrates**

The present invention also encompasses methods for identifying ADAMTS-SI substrates. Such methods include those wherein a candidate substrate is contacted with a polypeptide comprising an enzymatically active ADAMTS-SI polypeptide of the invention, and conversion of substrate to product is determined, or binding of polypeptide to the candidate substrate determined. The invention also encompasses rational drug design conducted using computer software that calculates interactions between candidate compounds and polypeptides or polynucleotides of the invention.

Substrates may be identified by a candidate protein or synthetic substrate approach. For example, candidate proteins can be cast within an agarose gel matrix and the ability of the ADAMTS-SI protein to digest the protein determined using protein zymography. See, P.D. Brown et al., *Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines*, 50(19) *Cancer Research* 6184 (October 1990). Alternatively, a phage display or fluorometric peptide library can be screened to identify substrates of the protein. See, D.R. O'Boyle et al., *Identification of a novel peptide substrate of HSV-1 protease using substrate phage display*, 236(2) *Virology* 338 (September 1997).

#### **Agonists/antagonists of ADAMTS-SI**

The present invention also includes assays for determining agonists and/or antagonists of ADAMTS-SI. Assays for determining aggrecanase, collagenase, procollagen protease and/or angiogenic activities may be used to identify agonist or antagonist compounds, preferably small molecular weight compounds of less than 700 daltons. The compounds may contain a hydroxamic acid moiety or an optionally substituted heterocyclic nucleus, or an aryl or heteroaryl sulfonamide moiety, which compounds inhibit or stimulate the

activity of endogenous or recombinant ADAMTS-SI. E.C. Arner et al., *Generation and characterization of aggrecanase. A soluble, cartilage-derived aggrecan-degrading activity*, 274 *Journal of Biological Chemistry* 6594 (March 1999); M.D. Tortorella et al., *supra*; A. Colige et al., *supra*; Vazquez et al., *supra*. ELISA or fluorescent substrate assays can be performed to determine agonists or antagonists, or to determine specific proteolytic activity, of an ADAMTS-SI protein.

#### Diagnostic Assays

The present invention also includes processes for diagnosing diseases or susceptibility to diseases related to expression and/or activity of ADAMTS-SI. Such diseases may be identified by determining the presence or absence of a mutation in the nucleotide sequence encoding said ADAMTS-SI polypeptide in the genome of a patient. Alternately, the presence or amount of ADAMTS-SI polypeptide in a sample derived from a patient may be determined as an indicator of disease or susceptibility to disease. Such diagnosis may be performed for diseases including the following: arthritis (osteoarthritis and rheumatoid arthritis), inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's disease, organ transplant toxicity and rejection, cachexia, allergy, cancer (such as solid tumor cancer including colon, breast, lung, prostate, brain and hematopoietic malignancies including leukemia and lymphoma), tissue ulcerations, restenosis, periodontal disease, epidermolysis bullosa, osteoporosis, loosening of artificial joints implants, atherosclerosis (including atherosclerotic plaque rupture), aortic aneurysm (including abdominal aortic and brain aortic aneurysm), congestive heart failure, myocardial infarction, stroke, cerebral ischemia, head trauma, spinal cord injury, neurodegenerative diseases (acute and chronic), autoimmune disorders, Huntington's disease, Parkinson's disease, migraine, depression, peripheral neuropathy, pain, cerebral amyloid angiopathy, nootropic or cognition enhancement, amyotrophic lateral sclerosis, multiple sclerosis, ocular angiogenesis, corneal injury, macular degeneration, abnormal wound healing, burns, diabetic shock, infertility and other diseases characterized by metalloproteinase activity and/or characterized by mammalian adamalysin activity.

#### Therapeutic compositions and uses of ADAMTS-SI

In one embodiment of the present invention, an antibody, agonist, antagonist, substrate and/or variant of ADAMTS-SI, or a polypeptide or polynucleotide of the invention, is employed in a therapeutic composition for treatment of arthritis (osteoarthritis and rheumatoid arthritis), inflammatory bowel disease, Crohn's disease, emphysema, acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, Alzheimer's disease, organ transplant toxicity and rejection, cachexia, allergy, cancer (such as solid tumor cancer including colon, breast, lung, prostate, brain and hematopoietic malignancies including

leukemia and lymphoma), tissue ulcerations, restenosis, periodontal disease, epidermolysis bullosa, osteoporosis, loosening of artificial joints implants, atherosclerosis (including atherosclerotic plaque rupture), aortic aneurysm (including abdominal aortic and brain aortic aneurysm), congestive heart failure, myocardial infarction, stroke, cerebral ischemia, head  
5 trauma, spinal cord injury, neurodegenerative diseases (acute and chronic), autoimmune disorders, Huntington's disease, Parkinson's disease, migraine, depression, peripheral neuropathy, pain, cerebral amyloid angiopathy, nootropic or cognition enhancement, amyotrophic lateral sclerosis, multiple sclerosis, ocular angiogenesis, corneal injury, macular degeneration, abnormal wound healing, burns, diabetic shock, infertility or other diseases  
10 characterized by metalloproteinase activity and/or mammalian adamalysin activity.

In one embodiment, polynucleotides of the invention can, for example, be employed to transform cells in gene therapy application, e.g., as part of in vivo or ex vivo gene therapy. Polynucleotides can also be employed in antisense therapy, and in the construction of ribozymes. Use of polynucleotides in these methods is known to those skilled in this art.

15 For administration to mammals, including humans, a variety of conventional routes may be used including oral, parenteral (e.g., intravenous, intramuscular or subcutaneous), buccal, anal and topical.

For oral administration, tablets containing various excipients such as microcrystalline cellulose, sodium citrate, calcium carbonate, dicalcium phosphate and glycine may be  
20 employed along with various disintegrants such as starch (and preferably corn, potato or tapioca starch), alginic acid and certain complex silicates, together with granulation binders like polyvinylpyrrolidone, sucrose, gelation and acacia. Additionally, lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc are often very useful for tableting purposes. Solid compositions of a similar type may also be employed as fillers in gelatin capsules;  
25 preferred materials in this connection also include lactose or milk sugar as well as high molecular weight polyethylene glycols. When aqueous suspensions and/or elixirs are desired for oral administration, the active ingredient may be combined with various sweetening or flavoring agents, coloring matter or dyes, and, if so desired, emulsifying and/or suspending agents as well, together with such diluents as water, ethanol, propylene glycol, glycerin and  
30 various like combinations thereof. In the case of animals, they are advantageously contained in an animal feed or drinking water in a concentration of 5-5000 ppm, preferably 25 to 500 ppm.

For parenteral administration (intramuscular, intraperitoneal, subcutaneous and intravenous use) a sterile injectable solution of the active ingredient is usually prepared. Solutions of the therapeutic compound in either sesame or peanut oil or in aqueous propylene  
35 glycol may be employed. The aqueous solutions should be suitably adjusted and buffered, preferably at a pH of greater than 8, if necessary and the liquid diluent first rendered isotonic. These aqueous solutions are suitable intravenous injection purposes. The oily solutions are

suitable for intraarticular, intramuscular and subcutaneous injection purposes. The preparation of all these solutions under sterile conditions is readily accomplished by standard pharmaceutical techniques well known to those skilled in the art. In the case of animals, compounds can be administered intramuscularly or subcutaneously at dosage levels of about 5 0.1 to 50 mg/kg/day, advantageously 0.2 to 10 mg/kg/day given in a single dose or up to 3 divided doses.

Active compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

10 For intranasal administration or administration by inhalation, active compounds are conveniently delivered in the form of a solution or suspension from a pump spray container that is squeezed or pumped by the patient or as an aerosol spray presentation from a pressurized container or a nebulizer, with the use of a suitable propellant, e.g., 15 dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurized container or nebulizer may contain a solution or suspension of the active compound. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated containing a powder mix of a compound of the invention and a suitable powder base such as 20 lactose or starch.

A therapeutic composition of the present invention can be administered to any subject having a medical disorder as herein described. Acceptable protocols by which to administer therapeutic compounds of the present invention in an effective manner vary according to individual dose size, number of doses, frequency of dose administration and mode of 25 administration. Determination of appropriate protocols is accomplished by those skilled in the art without undue experimentation. An effective dose refers to a dose capable of treating a subject for a medical disorder as described herein. Effective doses vary depending upon, for example, the therapeutic composition used, the medical disorder being treated and the size and type of recipient animal.

30 The dosage and length of treatment depends on the disease state being treated. The duration of treatment may be a day, a week or longer and may last over the lifetime of the patient.

#### **Identification of ADAMTS-SI**

35 For identification of novel ADAMTS gene family members, a non-redundant set of publicly available protein sequences was assembled (accession numbers: D67076, AJ003125, AB002364, AB014588). A series of low stringency BLAST searches were then performed against the LifeSeq Gold™ database (Incyte) using each of the above protein

sequences as queries and RecA-mediated homology capture used to isolate corresponding cDNA clones. Colonies hybridizing to the capture sequence were isolated and the largest clone (S3-2.6 kb) was sequenced. The sequence encoded a signal peptide, prodomain, furin cleavage site, and partial metalloproteinase domain. However, the Zinc binding motif, disintegrin, and thrombospondin motifs, were not present. Subsequently, attempts to clone ADAMTS family members led to the identification of a clone (I1A). Sequencing of this clone revealed 55 amino acids of overlapping sequence identity to ADAMTS-S and encoded the remaining metalloproteinase domain. Thus, by pursuing the cloning of two independent ESTs, the sequences collapsed into a single novel ADAMTS termed ADAMTS-SI (nucleotides 1-1385 of FIG.1 [SEQ ID NO: 1]). A published unannotated sequence (GenBank accession #AB037733) was found to overlap with our composite ADAMTS-SI clone and completed the identification of a full-length sequence for ADAMTS-SI [SEQ ID NO: 1]. Utilizing RT-PCR, the full-length sequence for ADAMTS-SI was cloned and sequenced in a series of overlapping fragments. The complete polypeptide sequence of ADAMTS-SI is shown in FIG. 2 [SEQ ID NO: 2]. Fragments comprising the nucleotides 1544-4480 of SEQ ID NO: 1 were amplified with the following primer sets from human osteoarthritic cartilage RNA: Nucleotides 1544-2516 of SEQ ID NO: 1 (sense 5'GGTGAATCCATCATGATACTGCTG3' [SEQ ID NO: 3]; antisense 5'GAAGCTGATAGTAGGCTGTGTTCCC3' [SEQ ID NO: 4]) Nucleotides 2171-4480 of SEQ ID NO: 1 (sense 5'ACAGATGGATCCTGGGGAAGTTGGA3' [SEQ ID NO: 5]; antisense 5'CTGACATACAACAACACGCCGCTGG3' [SEQ ID NO: 6]) Also, the 3' terminus comprising nucleotides 3596-5470 of SEQ ID NO: 1 was cloned by RT-PCR from A549 (human lung cell line) RNA with the following primer set: (sense 5'TCAGAGTGCTTGGTCACCTGT3' [SEQ ID NO: 7]; antisense 5'CCCGCTTTGCATACAAGGAA3' [SEQ ID NO. 8]). The RT-PCR performed to generate these overlapping fragments utilized the One-Step System (GIBCO, Gaithersburg,) according to the manufacturer's instructions with the addition of Elongase polymerase (Roche, Indianapolis, IN). Briefly, 1 ug of total RNA was used in each reaction containing 200 nM of sense and antisense gene-specific primers with 1 ul of Elongase polymerase added. The thermal cycling parameters were: 1 cycle at 50°C for 30 minutes, 94°C for 2 minutes, followed by 35 cycles of: 94°C 15 sec, 55°C 30 sec, and 72°C 6 minutes.

### **Expression of ADAMTS-SI in Chondrocytes Derived from Osteoarthritic (OA)**

#### **Cartilage and Induction by Proinflammatory Cytokines**

Primers to ADAMTS-SI were synthesized by Life Technologies (sense primer 5'-GAGAGCCTCAACAGGAGGC-3' [SEQ ID NO: 9 and antisense primer 5'-GCACTGAGTGAAAACCCATTCC-3') [SEQ ID NO: 10]. Chondrocytes were isolated from the cartilage of two patients with osteoarthritis and grown in tissue culture media. Chondrocytes were mock treated (lane 1 = patient 1, lane 3 = patient 2 in Figure 5) or treated



with retinoic acid (lane 2, patient 1 in Figure 5) or IL-1 (lane 4, patient 2 in Figure 5). RNA was isolated by standard methods and a 5 ug of each sample was used in a first strand cDNA synthesis reaction using oligo dT priming (using Superscript reverse transcriptase and conditions recommended by the manufacturer). 4% of the cDNA reaction products were used as template in a PCR reaction using PCR supermix (LTI) and 150 ng of the above primers using conditions recommended by the manufacturer. PCR cycling conditions 95°C 60 seconds followed by 30 cycles of: 94° C 30 seconds, 54° C 30 seconds, 72° C 45 seconds. Reaction products (10ul) were fractionated by agarose gel electrophoresis (4% NuSieve, FMC) and the ethidium bromide stained gel visualized by UV. PCR controls (water, lane 5 in Figure 5) and DNA molecular weight markers (not shown, PhiX phage DNA digested with HaeIII, LTI) were included. A product, indicated by the arrow and corresponding to the predicted size (~400 nucleotides) was observed in both patients and upregulated by treatment with proinflammatory agents.

#### **Expression and Purification of a Truncated ADAMTS-SI Fusion Protein**

A truncated version of ADAMTS-SI which included the pro, metalloprotease, disintegrin and first thrombospondin domains (amino acids 19-698 of SEQ. ID NO: 2) was cloned as a fusion protein with a heterologous amino-terminal secretory signal (from the human chitinase-like protein) and a carboxyl-terminal 6 histidine tag into a baculovirus expression vector (pFastBac derivative, GIBCO BRL, Gaithersburg, MD), and purified by nickel affinity chromatography and detected by Western blot using an antibody raised against the histidine tag epitope (Qiagen Inc., Valencia, CA). FIG. 6. The size of the expressed product (45 kD) was consistent with proper maturation of the protein by furin cleavage of the prodomain.

In particular, approximately 100 ml of conditioned insect cell media was dialyzed against PBS containing 20 mM imidazole and purified by batch affinity chromatography overnight at 4°C. The resin was washed with phosphate buffer containing 20 mM imidazole and eluted step-wise with phosphate buffer containing 50 mM, 100 mM, and finally 250 mM imidazole. Samples were diluted 2x with sample buffer (Novex, Carlsbad, CA) containing 5 % B-mercaptoethanol denatured by boiling for 2 minutes. 20 ul of each sample was resolved on 4-20% gradient SDS-PAGE mini-gel and transferred to Nitrocellulose (Novex, Carlsbad).

BLASTP 2.0.9 analysis showed high homology to a number of ADAMTS family members, as indicated in the alignment in Figure 4.

The foregoing description of the invention has been presented for purposes of illustration and description. Further, the description is not intended to limit the invention to the form disclosed herein. Consequently, variations and modifications commensurate with the above teachings, and the skill or knowledge in the relevant art are within the scope of the

present invention. It is intended that the appended claims be construed to include alternate embodiments to the extent permitted by the prior art.

#### **Expression and Purification of a Full Length ADAMTS-SI Fusion Protein**

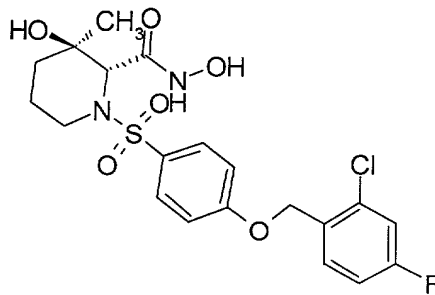
5 The full length ADAMTS-SI gene was cloned using RNA prepared from synovial tissue of a patient with osteoarthritic disease. The full length cDNA (nucleotides 413 ATG CAG TTT..... GAA CTG TAA 5302 of SEQ. ID NO. 1) was generated by RT-PCR and cloned into a mammalian expression vector, pcDNA3.1 TOPO-V5-His (Invitrogen). PCR and subcloning manipulations were then used to introduce a FLAG tag sequence immediately following the last 3' nucleotide of the coding sequence (nucleotide 5299 of SEQ. ID NO. 1).

10 HEK-293 (ATCC CRL-1573) cells (expressing large T antigen) were transfected with a nearly full length ADAMTS-SI V5/His construct (lacking the C-terminal 21 AA , SEQ. ID NO. 2, and the Flag tag at the carboxyl terminus) using Fugene6 reagent (Roche) according to the manufacturer's recommended protocol. Cells were incubated with transfection medium for ~12 hours, after which time the media was replaced with 17mls of IS293 serum-free media  
15 (Irvine Scientific) containing 2mM L-glutamine. At 24 hours, 50µg/ml soluble Heparin and 1.0µg/ml Aprotinin (Sigma) was added to the media. 48 hours post-transfection, media was collected, clarified and protease inhibitor tabs (Roche) added. Cells were collected and lysed in SDS protein sample buffer for western blot analysis. Expressed protein was purified by Ni-NTA Affinity Chromatography. The full length, Flag-tagged version of ADAMTS-SI was also  
20 expressed and purified (using Anti-Flag M2 Affinity Matrix).

Figure 7 shows the western blot of ADAMTS-SI expressed protein, probed with Anti-V5-HRP (Invitrogen). A band of ~180 kDa was detected in the cellular lysate and corresponds to the predicted size of the unprocessed pro form of the protein. A slightly smaller band (~147kDa) was purified from the media and corresponds to the secreted and  
25 furin processed mature form. In addition, the purified sample shows several smaller bands which may correspond to degraded and/or further processed protein.

#### **Aggrecanase Activity by ADAMTS-SI**

Recombinant human ADAMTS-SI (20 µl) and recombinant human ADAMTS-4 (MD Tortorella, *Science*, 284:1664) (20 µl) were incubated with 1 µM of aggrecanase substrate  
30 (aggrecan protein containing the interglobular domain (IGD)). The substrate is cleaved at the relevant aggrecanase site by aggrecanases such as ADAMTS-4. Other MMP enzymes such as matrix metalloproteinase-3 do not cleave at the aggrecanase site but instead cleave at a preferred metalloproteinase site that is N-terminal to the aggrecanase cleavage site. The aggrecanase substrate has been used to assess the activity of various aggrecanases, such  
35 as ADAMTS-4, and their inhibitors, such as compound I, shown below, and described in EP 1081137, published March 7, 2001:



Incubations were carried out in 20  $\mu$ l TSC buffer (50 mM Tris, pH 7.5 100 mM NaCl, 10 mM  $\text{CaCl}_2$ ) with protease inhibitor cocktail ("Complete", EDTA free from Roche: catalog number 1873580)  $\pm$  1  $\mu$ M compound I, for 18 hours at 37° C. The reactions were stopped by the addition of EDTA to 10 mM final concentration. The reaction mixtures were prepared for SDS-PAGE by the addition of 40  $\mu$ l 2x sample buffer (Novex) containing 0.5 M DTT and then separated on 4-20% Tris glycine gels (Novex). Proteins on the gels were transferred to a nitrocellulose membrane and the membrane blocked with 1x Western Blocking Reagent (Roche, catalog number, 1 921 673) in TTBS (100 mM Tris, pH 7.5, 0.9% NaCl and 0.1% Tween 20).

Treatment of the aggrecan protein with aggrecanases results in the formation of a characteristic cleavage site in the IGD. This produces a C-terminal neoepitope NITEGE-COOH, a signature for aggrecanase activity on aggrecan. A rabbit polyclonal antibody was prepared from rabbits immunized with a peptide (NITEGE-COOH) corresponding to the C-terminal neoepitope sequence. The crude rabbit antiserum was affinity purified against the corresponding neoepitope sequence and absorbed against a read-through peptide to ensure specificity for the neoepitope sequence. This affinity-purified antibody does not cross react with uncleaved substrate. Reactivity is only observed after cleavage at the characterized aggrecanase site revealing the NITEGE-COOH neoepitope and not at other sites such as a metalloproteinase site N-terminal to the aggrecanase cleavage site.

The blots prepared above were probed (1:1000 dilution) with the anti-NITEGE rabbit antibody. Following treatment with the anti-NITEGE antibody, the blots were washed and then treated with anti-rabbit IgG-HRP (1:1000) (Amersham, catalog number NA934). All antibody dilutions were in TTBS with 1x western blocking reagent. The antibody reactions were visualized with SuperSignal WestPico Chemiluminescent Substrate (Pierce, catalog number 34080) and viewed on a Lumi-Imager.

Figure 8 shows a Western Blot analysis of ADAMTS-4 and ADAMTS-SI cleavage of the aggrecanase substrate. These results showed that treatment of the substrate with both ADAMTS-4 and ADAMTS-SI resulted in cleavage at the previously characterized aggrecanase cleavage site, as revealed by reactivity with the specific neoepitope antibody. Furthermore, the activities of both enzymes were blocked by compound I, a compound that

has been identified as a potent inhibitor of IL-1 induced aggrecan degradation in human osteoarthritic cartilage explants ( $IC_{50} = 8.0$  nM), aggrecanase activity secreted by human osteoarthritic cartilage ( $IC_{50} = 4.1$  nM) and aggrecan degradation induced by intraarticular injection of IL-1 in hamsters (93% inhibition @ 30 mg/kg p.o.).

Preparation of the ...

Sequence ID Listing

SEQ ID NO: 1 is the nucleotide sequence of ADAMTS-SI.

SEQ ID NO: 2 is the deduced amino acid sequence of ADAMTS-SI.

5 SEQ ID NOS: 3-10 are nucleotide sequences of primers described in the Examples.

5  
10  
15  
20  
25  
30  
35  
40  
45  
50  
55  
60  
65  
70  
75  
80  
85  
90  
95  
100  
105  
110  
115  
120  
125  
130  
135  
140  
145  
150  
155  
160  
165  
170  
175  
180  
185  
190  
195  
200  
205  
210  
215  
220  
225  
230  
235  
240  
245  
250  
255  
260  
265  
270  
275  
280  
285  
290  
295  
300  
305  
310  
315  
320  
325  
330  
335  
340  
345  
350  
355  
360  
365  
370  
375  
380  
385  
390  
395  
400  
405  
410  
415  
420  
425  
430  
435  
440  
445  
450  
455  
460  
465  
470  
475  
480  
485  
490  
495  
500  
505  
510  
515  
520  
525  
530  
535  
540  
545  
550  
555  
560  
565  
570  
575  
580  
585  
590  
595  
600  
605  
610  
615  
620  
625  
630  
635  
640  
645  
650  
655  
660  
665  
670  
675  
680  
685  
690  
695  
700  
705  
710  
715  
720  
725  
730  
735  
740  
745  
750  
755  
760  
765  
770  
775  
780  
785  
790  
795  
800  
805  
810  
815  
820  
825  
830  
835  
840  
845  
850  
855  
860  
865  
870  
875  
880  
885  
890  
895  
900  
905  
910  
915  
920  
925  
930  
935  
940  
945  
950  
955  
960  
965  
970  
975  
980  
985  
990  
995